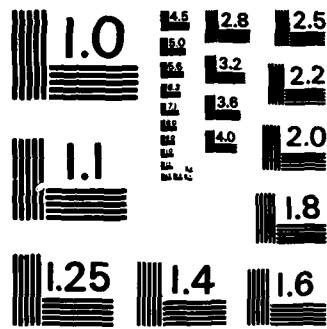


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with severity and intensity increasing as a function of both concentration of the fuel and duration of exposure. The alterations were manifested in the forms of cellular degradation, proliferation of membrane and non-membrane bound vacuoles, distortions of plasma membranes and a disruption of the structured association of cellular organelles. The integrity of plasma membranes directly contacting the fuel was maintained. Analysis of ruthenium red binding sites indicated that mucopolysaccharides adhere to surface epithelial cells of the nasal mucosa and gill throughout the duration of the experiment. ← →

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**AFOSR-IR- 82-1009**

**FINAL REPORT**

**THE EFFECTS OF JP-4 AVIATION FUEL ON**  
**SELECTED ORGANS OF THE FAT-HEAD MINNOW, PIMEPHALES PROMELUS:**  
**A CYTOCHEMICAL AND ULTRASTRUCTURAL INVESTIGATION**

**Sponsored by the**  
**AIR FORCE OFFICE OF SCIENTIFIC RESEARCH**

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**Research Location: Department of Biological Sciences,  
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PERTINENT INFORMATION CONCERNING  
THE RESEARCH PROJECT

A. Publications resulting from the research:

1. "Induction of Ultrastructural Alterations by Aromatic Hydrocarbons." 1982. Proceedings of the Electron Microscopy Society of America. 40: 54-55.
2. "The Induction of Ultrastructural Lesions in Selected Organs of the Fat-Head Minnow by Specific Aromatic Hydrocarbons." In preparation to be submitted to Virchows Arch. BB Cell Pathology.
3. "The Pseudobranch of the Fat-Head Minnow, Pimephales promelas: An Ultrastructural Investigation." In preparation to be submitted to The Journal of Morphology.

B. Personnel associated with research effort:

Lynn Kearns: Master's Degree candidate

Title of Thesis: "The Effects of JP-4 Jet Aviation Fuel on the Gill and Pseudobranch of the Fat-Head Minnow:  
A Histochemical and Ultrastructural Study"

Anticipated date of graduation: May, 1983

C. Presentations at scientific meetings and seminars

1. "The Effects of Aromatic Hydrocarbons on the Gill and Pseudobranch of the Fat-Head Minnow." Gulf Coast Research Institute. Ocean Springs, Mississippi. June, 1982. Seminar.
2. "Induction of Ultrastructural Alterations by Aromatic Hydrocarbons." Electron Microscopy Society of America meeting. Washington, D.C. August, 1982. Scientific meeting.

3. "The Effects of Aromatic Hydrocarbons on Mucopolysaccharide Distribution on Gill and Nasal Mucosa Surface Epithelial Cells." University of West Florida. Pensacola, Florida.  
November, 1982. Seminar.

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THE EFFECTS OF JP-4 AVIATION FUEL ON  
SELECTED ORGANS OF THE FAT-HEAD MINNOW, PIMEPHALES PROMELUS:  
A CYTOCHEMICAL AND ULTRASTRUCTURAL INVESTIGATION

by

William N. Norton

**ABSTRACT**

The primary objective of this investigation was to determine the acute ultrastructural effects of the water-soluble components of JP-4 aviation fuel on selected organs of the fat-head minnow, Pimephale promelus at the 20 and 60 percent concentration levels. The gill, pseudobranch, kidney and nasal mucosa from fish exposed to the fuel for 2, 12, 24 or 48 hours were excised and examined. Ultrastructural lesions were noted among all the organs analyzed with severity and intensity increasing as a function of both concentration of the fuel and duration of exposure. The alterations were manifested in the forms of cellular degradation, proliferation of membrane and non-membrane bound vacuoles, distortions of plasma membranes and a disruption of the structured association of cellular organelles. Analysis of ruthenium red binding sites indicated that mucopolysaccharides adhere to surface epithelial cells of the nasal mucosa and gill throughout the duration of the experiment.

#### **ACKNOWLEDGEMENT**

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## INTRODUCTION

There is a paucity of histopathological and ultrastructural information regarding specific responses of aquatic vertebrates to the aromatic hydrocarbons associated with petroleum derived aviation fuel. The water-soluble fraction of petroleum derived aviation fuel consists of a variety of aromatic compounds including toluene, benzene, xylene and naphthalene. Investigations have determined that the rate of aromatic hydrocarbon accumulation in aquatic organisms is highly species specific (Neff et al., 1976). Toluene is capable of accumulating in a variety of organs once uptake has occurred (Berry and Fisher, 1979). Histopathological investigations of mummichogs exposed to sub-lethal quantities of naphthalene indicate that the toxicity is due primarily to a generalized blood stasis (DiMichele and Taylor, 1978).

The present study represents a continuation of an investigation designed to determine the ultrastructural effects of the water-soluble fractions of JP-4 aviation fuel at the 40 percent concentration level on selected organs of the fat-head minnow, Pimephales promelas. Results of the latter study indicate that the water-soluble fractions of JP-4 are capable of inducing ultrastructural alterations in the kidney, gill, pseudobranch and nasal mucosa.

The primary objective of this study was to analyze the ultrastructural effects of relatively low and high concentrations (20 and 60 percent respectively) of the water-soluble fractions of JP-4 on selected organs of the fat-head minnow. In addition to conventional ultrastructural analysis, samples were also subjected to a preferential

cytochemical stain, ruthenium red, to determine if specific membrane-bound or surface related mucopolysaccharides were altered by components of the fuel.

## MATERIALS AND METHODS

Petroleum derived JP-4 was obtained from the Air Force Aero Propulsion Laboratory, Wright-Patterson Air Force Base, Ohio. The water soluble fraction was prepared by gently mixing a 95:5 ratio of water:JP-4 with a magnetic stirrer in a 9 liter stoppered glass container for 24 hours. The solution was allowed to stand for 24 to 48 hours before use. Little head space was allowed between the JP-4 floating on the water and the stopper. Concentrations of the water soluble fraction were expressed as micrograms of toluene, benzene and xylene per liter of water. The values were estimated to be about 87% of the total water soluble fraction (unpublished data). Separate calibration curves were generated from toluene, benzene, and o- and p-xylene. A stock calibration solution was prepared in methanol and kept under refrigeration.

Fat-head minnows of various weights were distributed into 3 groups of 12 fish each. Two groups were released into 20 gallon tanks containing 20 and 60 percent solutions, respectively, of the water soluble fractions of petroleum derived JP-4. The remaining group was maintained in a 20 gallon tank of water and functioned as a control.

At 2, 12, 24, and 48 hours subsequent to the initial exposure, 3 fish from each tank were removed and placed in a 2.5% glutaraldehyde-2% paraformaldehyde solution which was buffered with 0.1M sodium cacodylate (pH 7.4). To enhance the fixation of internal organs, a longitudinal slit was cut from the anus to the pectoral fins. Sections of gill, pseudobranch, nasal epithelium and kidney were excised and placed in vials containing cold fixative.

After 3 hours fixation, the tissues were rinsed in cold buffer and post-fixed in 2% osmium tetroxide for 2 hours. Subsequent to a rinse in buffer the tissues were dehydrated in a graded series of ethanol. Specimens to be analyzed by scanning electron microscopy were then critical point dried, metallically coated and observed in an ETEC Autoscan scanning electron microscope at 20 KV.

Organs to be further processed for transmission electron microscopy were embedded in Epon 812. Thin sections were cut by an ultramicrotome and then stained with lead citrate and uranyl acetate. The tissues were examined by a JEOL 100B transmission electron microscope at 60 KV.

Tissues to be analyzed for the presence of mucopolysaccharides were excised from the fish and placed in a 1.2% glutaraldehyde solution containing 0.05% ruthenium red. The solution was buffered with 0.067M sodium cacodylate (pH 7.2). The tissues were then rinsed in buffer and post-fixed in osmium tetroxide. Subsequent to dehydration in a graded series of ethanol, the specimens were embedded in Epon 812, sectioned and observed by the transmission electron microscope.

## RESULTS

Although the acute ultrastructural effects of the water soluble fractions of JP-4 appeared to be related temporally in regard to severity and extent of cytological damage, the various manifestations of exposure were evident even at the shortest time period investigated and at the lowest concentration of fuel components. Pronounced modifications of behavior were noted as soon as the fish were immersed in the fuel. The normal pattern of swimming as an organized school or group was altered as the fish seemingly attempted to avoid contact with one another. The experimental organisms also displayed erratic swimming patterns with tendencies to surface repeatedly.

### Gill:

Cells comprising the gill filaments and secondary lamellae were analyzed during the investigation. The surface junctional sites of adjacent control cells associated with both filaments and lamellae were raised in the form of micro-ridges. At various sites the ridge formations were disrupted and manifested in the form of micro-projections which were similar in appearance and dimensions to those protruding from the cellular surfaces. Both filaments and secondary lamellae of experimental organisms displayed altered surface junctional sites as exemplified by the formation of non-uniform blebs and a reduction in the size and elevation of the ridges. The micro-projections evident on the surface of control cells were significantly reduced or in some instances totally absent among experimental cells, resulting in a smoother contour.

Focal sites of surface epithelial cell degradation was noted among the secondary lamellae. The majority of cells which appeared in various stages of alteration contained membrane-bound vacuoles and myeloid figures. Also noted were instances where significant regions of epithelial cells protruded from the surface of secondary lamellae. There was no indication of fragmentation or dissociation of plasma membranes directly contacting the fuel.

Pillar cells, whose lateral extinctions normally form the smooth inner surface of capillaries within the secondary lamellae, displayed no abnormalities in regard to cytoplasmic organelles. However, membranes lining the lumen of several capillaries of experimental fish appeared distended and irregular in appearance. There was some indication of a disruption or dissociation of the basal lamina located between the pillar and epithelial cells.

Macrophages were observed within the gill filaments and secondary lamellae, however, as compared to control tissue the concentration appeared to be within normal ranges. Leukocytes and erythrocytes evident in the capillaries of sectioned filaments and lamellae displayed no signs of cellular alteration.

There appears to be little difference among surface epithelial cells of the gill filaments and secondary lamellae of experimental and control fish in regard to the distribution of mucopolysaccharides as monitored by ruthenium red binding. The results indicated that mucopolysaccharide substances were sustained on the surface of gill filaments and secondary lamellae of all experimental fish throughout the duration of exposure. The integrity of junctional complexes was maintained as indicated by the fact that ruthenium red-mucoid complexes were not evident in the intercellular spaces of the filaments

and lamellae. There appeared to be no distinct pattern of mucopolysaccharide distribution. The various sites of relatively high concentrations of the mucoid substances were not localized to the surfaces of a particular cell type.

Pseudobranch:

The outer surface of the control pseudobranch, attached to the anterior wall of the gill chamber, displayed ridge-like elevations at the junctions of adjacent cells. In contrast to the junctional complexes associated with cells comprising the gill filaments and secondary lamellae, the micro-ridges of the pseudobranch demonstrated a greater continuity with less disruption of the ridge-like pattern. Micro-ridges of various lengths and patterns were also evident along the cellular surfaces. The integrity of junctional ridges was maintained in experimental organisms, however, the cellular surface elevations were altered in regard to concentration and height.

The primary cell type of the pseudobranch appeared uniquely structured with an ordered arrangement of tubules which were tightly grouped both together and in conjunction with elongated mitochondria. The tubular network evolves from invaginations of the plasma membrane which contacts the basal lamina positioned adjacent to the capillary systems of the pseudobranch. The fuel exerted a disruptive effect on the structured system of mitochondria and tubules. In essence, the latter structures were unable to maintain the close association noted in control tissue.

Vacuoles, both membrane and non-membrane bound, were evident in a majority of the cells examined from experimental tissue. The vacuoles of various shapes and dimensions appeared to alter nuclear

morphology as well as tubular organization. Occasional mitochondrial degradation was manifested in the form of distensions of the outer membrane, and in some instances by the development of myeloid figures. Although morphometric analyses were not conducted, random scans of pseudobranch indicated that substantial numbers of cells were at least partially affected by the fuel.

There was no evidence of alteration or dissociation of the endothelial lining of the capillaries associated with the pseudobranch. Erythrocytes and leukocytes observed in the latter vessels appeared normal.

Nasal Mucosa:

Surface projections of the cells comprising the nasal mucosa were present in the form of cilia and microvilli. As compared to the control, cellular projections of experimental tissue revealed discernable alterations. Specific regions also displayed apparent reductions of both cilia and microvilli. Blebs were noted on the surface of cilia in experimental organs. Cellular junctions appeared unaltered by exposure to the hydrocarbons. The integrity of plasma membranes directly contacting the fuel was maintained. The underlying epithelial cells of the control nasal mucosa were characterized by cytoplasms of varying electron densities, however, no evidence of cellular degradation was noted.

Cellular abasement, manifested by the formation of myeloid figures, electron dense bodies, and organelle disruption was evident throughout the epithelial region of experimental tissues. The predominance of cellular lesions occurred in association with basal and supportive epithelium. There were relatively few

observations of cellular degradation among sensory receptors. Mucoid cells appeared to be unaffected by components of the fuel.

Ruthenium red binding indicated that mucopolysaccharides coat the outer membranes, including cilia, of surface epithelial of the nasal mucosa throughout the duration of exposure to the fuel. There appears to be no significant difference in the thickness of the mucoid coat among experimental and control cells.

Kidney:

Cells comprising the proximal convoluted tubules of experimental fish appeared to possess a greater number of apical vacuoles as compared to the control. Leukocytes and erythrocytes present within the capillaries of experimental organisms appeared normal. Vacuoles of various dimensions were observed in association with the endoplasmic reticulum of cells comprising the distal convoluted tubules. No ultrastructural alterations were detected among the podocytes, fenestrated endothelium or basal lamina associated with the glomerulus.

Endothelial cells normally adjacent to the basal laminae of proximal and distal convoluted tubules were occasionally observed in a state of dissociation. Plasma membranes of the aforementioned cells appeared fragmented with a resulting exposure of the basal lamina to the peritubular vessels. At the sites of alteration some disruption or dissociation of the basal lamina was noted.

## DISCUSSION

Results from the present investigation indicate that the aromatic hydrocarbons comprising the water soluble fractions of petroleum derived JP-4 fuel at the 20 and 60 percent exposure levels induce ultrastructural alterations among the tissues examined. The cellular lesions, manifested in a multiplicity of forms, are consistent in regard to their presence throughout the study. However, severity as determined by the extent of cellular damage gradually increases as the exposure period progresses and the concentration of the fuel components increases. While the range and severity of lesions appears to be a function of the length of exposure, unresolved is whether the progressive damage is due to a consistent uptake and accumulation of the chemicals or to an initial irreversible exposure. Although a gradual reduction in the aromatic hydrocarbon content of the experimental system occurs during the investigation, the study is not designed to determine if the reduction is due to evaporation or a continual uptake by the fish or a combination of both. That aquatic organisms are capable of accumulating aromatic hydrocarbons has been documented (Neff et al., 1976; Benvile and Korn, 1977). However, little ultrastructural and histopathological information is available concerning the adverse effects of the aromatic hydrocarbons analyzed in the present investigation to specific species of fresh water fish.

Although a variety of cytoplasmic modifications are evident, the ultrastructural integrity of plasma membranes directly contacting the water soluble fractions of the fuel is maintained. Thus, the aromatic hydrocarbons apparently do not exert their observable

effects upon biological membranes in the capacity of an organic solvent.

The dissociation of mitochondria-tubule complexes, which are characteristic of cells comprising the pseudobranch may be the result of an alteration of either membrane-bound or soluble binding factors. The sites of alteration appear in association with and devoid of vacuole formation. The disruptions of intracellular organization do not result in pronounced degradation of organelles, at least during acute exposures of the hydrocarbons analyzed during the present investigation. There were relatively few observations of cells in the latter stages of degradation which is usually characterized by the presence of multiple myeloid figures and massive disruption of organelles. The close proximity of mitochondria to the tubular network indicates that the association is required for a particular biochemical process, and that the disruptive nature of the fuel would adversely affect normal biochemical activities.

The function of the pseudobranch is still a matter of speculation. Since the structure receives oxygenated blood only, it cannot function in respiratory gas exchange. The pseudobranch is suspected to be involved in the metabolic gas exchange of the retina and possibly the regulation of gas into the gas bladders of certain species (Bond, 1979). The pseudobranch contains higher concentrations of carbonic anhydrase enzymes than any other organ of the body, and thus is capable of facilitating the dissociation of carbonic acid into carbon dioxide and water (Laurent, 1977).

Based upon ultrastructural observations of the nasal mucosa, cellular sensitivity to aromatic hydrocarbons appears to be selective. Degradation of basal and supportive epithelial cells is extensive, however, instances of sensory cell alteration is less evident. In addition, cellular degradation is more pronounced in the underlying epithelial layers of the mucosa. Although lesions in this area would not prove lethal, the degeneration may significantly impair the ability of the fish to respond to chemicals within the aquatic environment.

Gill structure appears to be very sensitive to the hydrocarbon constituents of petroleum. The effects are manifested by the formation of cellular lesions, hyperplasia, edema and fusion of secondary lamella (Woodward et al., 1981; Nuwayhid et al., 1980; Hawkes, 1977). Aromatic hydrocarbons associated with JP-4 are capable of inducing observable alterations among the epithelial cells of the gill. A reduction in the number of micro-projections and altered surface junctional sites among cells that comprise the surface of the gill indicate the detection and subsequent responses by the latter organ to the presence of a toxic chemical. Exposures of manganese to the fat-head minnow have resulted in similar surface modifications of the gill filament (Hinton and Walker, 1980).

Within the secondary lamellae sites of alteration along the plasma membranes of pillar cells forming the inner-lining of capillaries are expressed as invaginations and folds. The effect of the latter on functional capabilities of the gill is difficult to ascertain, however, chemical-induced changes in gill structure can have significant effects on gas exchange, osmoregulation and

susceptibility to disease (Hodgins et al., 1977). Undetected during ultrastructural observations of the present investigation were fused secondary lamellae which have been reported in fish exposed to a variety of hydrocarbons at various concentrations (Woodward et al., 1981; Hawkes, 1977).

Proliferation of macrophages within the secondary lamellae of fat-head minnows can be induced by exposure to ferric oxide (Hinton and Walker, 1980). Macrophages are occasionally noted in the lamellar subepithelial spaces of experimental fish, however, the same cellular arrangement is evident in control gills. Plasma membranes forming the surface of gill filaments and lamellae do not dissociate or fragment as a result of direct contact with the fuel.

Acute exposure of JP-4 to the kidney appears to alter selectively the endothelial cells associated with the distal and proximal convoluted tubules. This phenomenon indicates a lack of uniformity or distinct pattern in regard to the observable effects of the aromatic hydrocarbons on endothelial cells as a whole. Comparable cells of the gill, as previously mentioned, are adversely affected whereas those of the pseudobranch display no susceptibility to the fuel. Concomitant with the disruption of endothelial cells associated with tubules of the kidney are focal sites of basal lamina dissociation. Ultrastructural lesions of the basal lamina appear to be restricted to the tubules, since similar structures affiliated with the glomeruli display a normal morphology.

Results of ruthenium red binding indicate that mucopolysaccharides are present on the surface of epithelial cells and their associated

projections of the nasal mucosa and gill filaments and secondary lamellae. The observations imply that mucoid cells function in releasing mucopolysaccharides concomitant with exposure to the fuel components. The cytochemical evaluation also indicates that membrane integrity is maintained since there appeared to be no seepage of the ruthenium red-mucoid complex into the cells or cell surface modifications. Junctional complexes were also maintained as indicated by an absence of the ruthenium red in intercellular spaces.

In summary, the aromatic hydrocarbon components of JP-4 at the 20 and 60 percent exposure levels are capable of inducing ultra-structural lesions in organs selected for this investigation. The extent and severity of cellular alteration is directly correlated to the length of exposure and concentration of the water-soluble fraction of the fuel. The effects span a wide spectrum of observable alterations including the modification of surface structures, the degradation of cells, the proliferation of vacuoles and a disruption of the structured association of cellular organelles.

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